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REVIEW ARTICLE

Problems of the Qualitative and Quantitative Analysis of Plant Volatiles

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Abstract—Strategies for the analysis of plant volatiles have changed significantly over the past 15–20 years due to the introduction of new approaches to sample preparation and analysis, including those initially developed for other areas and currently applied to the analysis of plant metabolites. Any analysis of plant substances consists of two phases. The first phase includes plant material collection, primary processing, conservation, storage, and extraction to prepare samples for research. The second phase is the analysis itself by various chromatographic, spectral, and/or hybrid (hyphenated) techniques. Most scientific publications focus their attention on the second phase, and the first remains "behind the scenes," although it is in the first phase that the biomaterial experiences significant transformations. It is impossible to correctly and adequately evaluate the ultimate result of a study without taking these transformations into account. Specific difficulties arise in both phases, and they are reviewed in this paper. The wide distribution of modern chromatographic instruments equipped with sophisticated software allows a significant portion of an experiment to be performed automatically. However, one should realize that the improvement of experimental techniques does not change the basics of a method, and, therefore, does not eliminate its intrinsic limitations. To avoid fallacies in the publication of the results, all the experimental data obtained in the automatic mode should be subjected to an impartial revision by the experimenter with regard to all known limitations inherent in methods used for separation and detection of components. In order to correctly interpret experimental results, one should know the entire history of samples under investigation; thus, it is necessary to document carefully all manipulations with plant material from the collection of raw materials till the final sample preparation. Only with this proviso the study can be expected to provide meaningful results.

Keywords: plant volatiles, gas chromatography, gas chromatography–mass spectrometry, qualitative analysis, quantitative analysis, detectors, standards, artifacts

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CONTENTS

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INTRODUCTION

Plants produce, accumulate, and release volatile substances, which are a heterogeneous group of low-molecular-weight molecules with saturated, unsaturated, linear, branched, and cyclic fragments. They bear various chemical groups: hydroxy, carbonyl, ester, carboxy, etc. Terpenoids (isoprenoids) constitute the largest subgroup of volatile plant metabolites, including over 40000 structures [1]. The main biochemical pathways of the synthesis of volatiles have been identified, and many genes encoding the appropriate enzymes are known [2]. Numerous volatile substances are used in the food, pharmaceutical, agricultural, and chemical industries. Special attention is placed on small molecules that play key roles in biology, acting as intermediates in many important processes: metabolism, signal transduction, mating, and chemical protection. Small molecules are classified with traditional categories: metabolites, secondary metabolites, pheromones, hormones, etc. Some of these categories overlap, and more than one keyword may be attributed to a single compound. For this reason, the umbrella term *small biogenic molecules* is in increasing use for description of any small molecule derived from a biologic source. Knowledge of the structures and relative abundances of volatiles released by plants is of great significance for many basic and applied fields in biology, chemistry, and interdisciplinary research. To obtain such information, scientists investigate profiles of plant volatiles and overcome analytical difficulties stemming from the fact that they deal with complex mixtures of components with diverse structures and properties. The chemistry of natural products and metabolomics are two major disciplines concerning the chemical profiling of plant objects [3]. The study of plant volatiles requires such analytical methods and technologies that would allow not only the assessment of volatile substance composition but also the monitoring of the variation of their profiles and the detection of traces of substances characteristic of target plant species. Analytical strategies have changed profoundly over the past 15–20 years because of the introduction of new approaches to sample processing and analysis. Some of these approaches were designed for other fields of inquiry, and now they are applied to the analysis of plant volatiles. The development of analytical methods and their impact on the strategies of plant volatile studies are concisely reviewed in [4–7].

Any analysis of plant substances includes two phases. The first phase includes collection, primary processing, preservation, storage, and extraction to prepare samples for the second phase, the analysis proper. In the second phase, the prepared samples are analyzed by chromatographic, spectrometric, and/or hyphenated methods. In many publications, attention is focused on the second phase, whereas processes occurring in samples in the first phase are behind the scenes. However, these processes are significant, and the ultimate result of the study cannot be properly interpreted without their consideration. Specific problems arising in both phases of a study are the subject of this paper. The first cluster of problems is considered in the section *Native components or artifacts?*, and the second, in subsequent sections *Qualitative analysis*, *Quantitative analysis*, and *Analysis of specific types of substances*.

The first procedures carried out by a student of plant metabolites are the isolation and concentration of plant substances, many of which are highly labile, as they tend to undergo various chemical processes outside plant tissues. At present, no methods for qualitative or quantitative analysis of substances directly in intact plant tissues are known, and any method of isolation and concentration either distorts the proportions of native components or produces artifacts, i.e., substances originally absent from the matter under study and derived from native substances in the course of extraction and sample pretreatment.

NATIVE COMPONENTS OR ARTIFACTS?

Artifacts in plant material analysis stem from two causes: experimental inaccuracy and the instability of analytes under experimental conditions.

To avoid experimental flaws, it is necessary to invoke the experience of other scientists and to understand the main causes of the flaws. A compendium of traps and problems encountered by all analysts using various spectrometrical and chromatographic methods (GLC, mass spectrometry, HPLC, and TLC) has been published and dedicated to "the innumerable scientists who made mistakes, used impure chemicals and solvents, suffered the consequences of unanticipated side-reactions, and were otherwise exposed to mayhem yet were not too embarrassed to publish their findings" [8].

However, even with analyses done thoroughly and all manipulations prescribed for isolation of plant metabolites [9] followed painstakingly, artifacts can be unavoidable, and labile native compounds undergo chemical transformations at various steps of the study: (1) plant material pretreatment (collection, transportation, drying, crushing, etc.), (2) isolation (extraction) of substances from the raw material, (3) extract storage, and (4) analytical manipulations.

Plant Material Pretreatment

Plants are injured when being cut. As a result, the normal plant life is disturbed and stress-associated

metabolites, i.e., substances normally absent from the plant, are produced [10, p. 19]. The gradual ebb of all vital functions during plant material drying disrupts the balance of normal biosynthetic processes and causes enzymatic conversions of native components not characteristic of a living plant. The profile of natural compounds in dried material changes with time, although all enzymatic processes are arrested by desiccation. In this situation, the secondary processes include loss of the most volatile components and conversions of plant components driven by light and the oxidative potential of aerial oxygen diffusing into tissues. For instance, the study of dill oil samples obtained from freshly collected plants, plants air-dried in the common way, and lyophilized plants revealed the appearance of artifacts and disappearance of some native component. Air-dried dill yielded ten times less oil than fresh plants, and the oil nearly completely lost benzofuran derivatives, the most important olfactive components of dill [11]. Examples of differences among volatile profiles of various wild plants stored for different time spans are presented in [12]. Differences of metabolic profiles of juniper (*Juniperus monosperma* (Engelm.) Sarg.) in different processing protocols are reported in [13]. The protocols were as follows: (A) Immediately after cutting, plants were placed on dry ice for 5 h and then stored at −80°C for three weeks (control). (B) Plants were kept at room temperature for 24 h, frozen to −80°C, and stored at this temperature for three weeks. (C) Plants were kept at room temperature for the first 24 h and then kept at +8°C for three weeks. The study showed that juniper shoots should be stored for no more than three weeks after cutting, and the storage temperature should be below +8°C; otherwise, terpenoid profiles are considerably distorted as compared to the control.

Freshly cut plant material can be used in studies of volatiles, but such studies demand that steam distillation and all preliminary manipulations be carried out directly at the sampling site under field conditions [14].

In practice, it is difficult to arrange field work so that plant material or fresh extracts could be preserved. Therefore, most scientists settle for work with dried material and knowingly deal with altered metabolite compositions, containing secondary products and artifacts.

Extraction

Isolation of labile native substances faces many problems arising during extraction and analysis. Various separation protocols involving certain methods or combinations are discussed in [15]. Specific features of conventional methods for substance isolation from plants inevitably generate artifacts [9]. Peroxides are a significant group of natural substances [16, 17], and many of them are important bioactive molecules [18–20]. Problems arising in the isolation and analysis of these substances are associated with their instability.

Liquid extraction. Generally, liquid extraction is applied to dried material, where all membranes have lost their original properties or are completely destroyed. Extraction with weakly polar organic solvents (hexane, petroleum ether, gasoline, aromatic and chlorinated aliphatic hydrocarbons, or benzene) affects native components mildly, whereas alcohols and aqueous organic solvents can modify some of them. The main difficulties in the latter case are associated with partial hydrolysis of some groups of substances (esters and glycosides of various structures), esterification, and interesterification [9, 21–23]. Specific problems are made by certain admixtures, particularly, in chlorinated organic solvents. Their use generates several known types of artifacts [9].

Steam distillation. Steam distillation is the simplest and most widespread method for isolating essential oils. It is commonly known that some essence components are absent from intact plants; rather, they are extraction artifacts. They can be formed by enzymatic degradation or chemical decomposition occurring in heating with the presence of water. These processes are associated with degradation of labile and high-molecular-weight compounds. Plant tissues contain certain amounts of lower organic acids; hence, steam distillation always occurs under weakly acidic conditions, which adversely affect the preservation of native components. Linalool is not decomposed in the pH range from 5.0 to 8.0, but the allylic rearrangement of linalyl acetate readily occurs at pH 7.0, not to speak of weakly acidic settings. Therefore, materials containing linalyl acetate are always difficult to analyze because of artifacts [24]. Essential oil components are often produced by hydrolysis of corresponding glycosides, as in the cases of rose oil [25] and many other essences [26–30].

In steam distillation, native components of plant material are extracted owing to diffusion or released in the degradation of special secretory bodies [14, pp. 19–26]. Most often, volatiles are found in glandular hairs. These hairs are bodies diverse in morphology [31], whose roles are the synthesis and secretion of certain substances [32], not necessarily volatile [33].

Direct sampling from secretory bodies and analysis of the matter has revealed dramatic differences in the qualitative and quantitative compositions between essential oils obtained by steam distillation, on the one hand, and the content of glandular hairs, on the other hand [34, 35]. The conclusion drawn in the studies is disappointing: the analysis of essential oils is basically the analysis of artifacts formed in distillation and gas chromatography procedures.

The main pathways generating artifacts are associated with thermal, hydrolytic, and oxidative processes and with light-induced and acid-catalyzed transformations during extraction. The major groups of artifacts are chamazulene and derivatives; spathulenol [36], products of hydrolysis of phospholipids and glycosides, and products of the resolution of chlorophyll

and cuticle biopolymers. The last group includes cutin [37], cutan [38], and other epicuticular components [39, 40]. The main pathways of artifact formation and the major groups of artifacts are discussed in the guidebook on the analysis of plant volatiles [10, pp. 194–220]. Known artifacts belonging to various structural groups are mentioned in the book on essences [41, pp. 7, 45, 53, 145, 175, 182].

Many artifacts belonging to terpene hydroperoxides are found in terpene-containing materials. Various monoterpenes are employed in beauty care products and perfumery to add certain fragrances. However, the most frequently used terpenes are readily oxidized when exposed to air to form hydroperoxides, which are potent skin sensitizers [42]. Allergic activity has been proven for oxidized derivatives of linalool [43–45], linalyl acetate [46, 47], limonene [48–52], cinnamic alcohol [53], and citronellol [54]. In a study of lavender oil, the rates of the formation of allergenic hydroperoxides were found to be the same in the oxidation of free terpenes or terpenes within the essence [55].

Extract Storage

Within the time span between the extraction and analysis of the extracted matter, the composition of the latter may suffer changes whose type and degree depend on storage conditions. These changes have been best investigated in essential oils, many of which are commercially available [41]. Therefore, the preservation of their composition and properties is important in practice.

Essential oils are unstable by their nature. They undergo spontaneous chemical transformation, which eventually alter their chemical composition. Changes in the volatile profiles and formation of artifacts during essence storage were demonstrated for citrus oils [56, 57]. When stored in the cold (–21°C) within 12 months, citrus oils showed no significant changes, but after exposure to room temperature (+20.5°C) the proportions of components changed profoundly, and numerous artifacts emerged. In particular, 34 artifacts were found in oil from *Citrus aurantium*, and they constituted 17% of all volatiles [57]. The most abundant artifacts were (+)-carvone, *trans,trans*-farnesyl acetate, sabinene hydrate, 1-octene-2-ol, *cis,cis*-farnesyl acetate, and dihydrocarveol acetate. The chemical composition of essence from pomelo (*Citrus maxima*) was studied in [58] in connection with the deterioration of its fragrance with storage. It was shown that the unpleasant notes emerging in storage were associated with the formation of oxidized linalool and limonene derivatives, caused primarily by the concurrent action of air oxygen and sunlight. The most appropriate sets of test parameters for tracing chemical changes during essence storage are discussed in [59]. Current knowledge of possible changes in essential oils and factors affecting essence stability is comprehensively reviewed in [60]. The review describes various pathways of degradation caused by ambient factors and analytical methods for assessment of both native and altered essence profiles. The key role in the formation of artifacts in storage is thought to be played by oxidative processes. Part of them can be inhibited by adding antioxidants [61].

According to our experience, the compositions of the overwhelming majority of essence samples kept in darkness in vacuum-sealed ampoules at 5–8°C remain unchanged for years[1]. The most notable exceptions are samples of essential oils containing significant amounts of organic acids. In such cases, even with proper preparation and storage, the samples undergo diverse acid-induced transformations distorting the native composition.

Analysis

Some components of plant extracts are stable enough during isolation but are too thermolabile to tolerate common GC analysis. The Cope rearrangement of bicyclogermacrene to bicycloelemene at heating has long been known [62]. Germacrane derivatives are generally thermolabile, and they undergo various reactions during gas chromatography [63, 64], hampering their quantitation [65].

Hydroperoxides are also too thermolabile to survive GC. For this reason, Nilsson et al. [66] compared various ionization methods in hydroperoxide detection by liquid chromatography–mass spectrometry (LC–MS). They showed that ionization efficiency was substantially influenced by mobile phase composition and device tuning.

The problem of thermolability can be coped with to an extent by so-called cold injection to the GC column, which allows avoiding the heat stress caused by heating in the evaporator [34].

An appropriate alternative is NMR spectrometry. It allows the detection and quantitation of thermolabile components.

QUALITATIVE ANALYSIS

The state of the art in qualitative chromatographic analysis is reviewed in [67]. Gas chromatography is the first-line method for analyzing complex mixtures of biogenic volatiles, such as floral aromas and essential oils [68]. Gas-chromatographic analysis of essential oils is briefly outlined in [69].

As gas chromatography is a widespread technique in routine analyses of essences, the protocols should be optimized, and their duration should be as short as possible. The Fast-GC method, described in [6, 70, 71], reduces analysis duration nearly tenfold, to 3–4 min in comparison to 30–40 min in the conventional

[1] This is true only for properly prepared samples, carefully dried and free of desiccant remnants.

analysis, by using short capillary columns (about 10 m in length) and fast heating, up to 40°/min.

Retention Indices

Retention is one of the main parameters characterizing the behavior of a substance in chromatographic analysis. The retention index is its generally accepted measure. The isothermal and linear temperature-programmed modes demand different procedures for calculating retention indices. Retention in the isothermal mode is characterized by Kováts retention indices [72]. Kováts indices vary with temperature [73] and stationary phase type. At present, the mean interlaboratory reproducibility of Kováts indices is about 5–10 units for standard nonpolar phases and 10–25 units for standard polar ones [74]. In linear temperature programming, retention times of homologs linearly increase with the number of carbon atoms in the molecule. Therefore, linear interpolation and absolute retention times can be used. Retention indices in the linear temperature-programmed mode are called linear retention indices (LRIs). Their calculation is simpler than in case of Kováts indices [75]. Linear retention indices are widely employed in the identification of volatile components of plant extracts, and the limits of their applicability are considered in technical studies [76]. Retention indices deduced from experiments conducted in the linear programmed mode depend on the temperature programming profile. It should be kept in mind that isothermal Kováts indices and retention indices obtained in the temperature-programmed mode are incommensurable and nonproportional and that LRIs measured in different temperature regimes may differ greatly [10, pp. 89–90]. Therefore, data reported in the literature should be used with due circumspection.

In the guideline method of obtaining reproducible profiles (fingerprints) of volatiles in GC on nonpolar stationary phases, a chromatogram is recorded under linear temperature programming at the rate 4°C/min within 50–250°C [77]. Retention indices for the HP-5ms column reported in the manual [10] were measured just in these settings. There are other retention index libraries, e.g., the library for the DB-5 column at the heating rate 3°C/min within 60–246°C [78] and the library for the MassFinder 4 program, which reports retention indices for the DB-1 column with temperature programming regimes not indicated.

Features of retention indices obtained in the temperature-programmed mode and their correlation with thermodynamic indices of components are discussed in [79]. The procedure of LRI determination in the analysis of plant volatiles is described in [10, pp. 78–91].

Determination of retention indices is the first step in the GC identification of substances in relatively simple mixtures. Analysis of more complex mixtures with multiple overlaps of chromatographic peaks demands more nuanced approaches. One of them is two-dimensional gas chromatography (GC × GC, or 2D GC) [80]. The tooling backup of 2D GC determines certain features in the analysis and interpretation of retention indices. A special analysis design involving methods for constructing retention correlation maps for the first and second dimensions has been developed. The first dimension generates linear temperature-programmed indices, whereas the second dimension may be interpreted in terms of "pseudoisothermal" retention indices [81].

Retention index values are unknown for many components of complex native mixtures; therefore, predictions of the chromatographic behavior of individual components are needed [82]. Earlier studies showed that retention indices of aroma substances in GC on nonpolar and polar stationary phases could be predicted within 3.6 and 5.6%, respectively [83]. Approaches to the prediction of retention parameters in terms of QSAR[2] models are being developed [84]. The association between molecular structures and retention parameters in various chromatographic techniques is discussed in [74], and it is indicated that the choice of descriptor sets is most important, because not all physicochemical descriptors correlate satisfactorily with retention parameters, and the simplest design parameter, formation heat, is not associated with chromatographic retention. The most complete collection of molecular descriptors is a comprehensive review of this field of research from its nascence to the present. The practically oriented reference book gives a thorough overview of different molecular descriptor representations and their corresponding molecular descriptors [85]. The quality of index prediction worsens as the stationary phase polarity increases [74]. As shown by examples of alcohols and esters, retention times can be predicted for certain stationary phases at temperatures within 70–140°C on the basis of molecular descriptors implemented in CODESSA™[3] software [86].

Mass Spectra

Gas chromatography with mass-spectrometrical detection (GS-MS) is the commonest method of investigation and analysis of volatile and conditionally volatile secondary plant metabolites [68]. Gas chromatography combined with mass spectrometry–electron impact ionization (EI-MS) has gained wide acceptance in recent decades and become a routine method for analysis of volatile and conditionally volatile substances. The mass spectra it produces are an efficient tool in the identification of components in complex mixtures. However, the application of the

[2] **Q**uantitative **S**tructure-**A**ctivity **R**elationship.

[3] **Co**mprehensive **D**escriptors for **S**tructural and **S**tatistical **A**nalysis (http://www.semichem.com/codessa/).

MS detector in gas chromatography faces a number of difficulties, first of all, the limited reproducibility of EI–MS spectra in GC–MS. Mass spectra of the same substance obtained by electron impact at the standard ionizing electron energy 70 V in different devices may differ considerably [87]. Moreover, in the same device mass spectra may differ even when recorded under strictly controlled conditions. The poor reproducibility is related, first, to intrinsic features of mass spectrometry itself [88], and second, to engineering and operational features of GC-MS as a hybrid method:

—Relative fragmentation rates along different pathways may vary with ion source temperature to impair the relative peak amplitude reproducibility in the resulting mass spectrum.

—The pressure in the ion source generally exceeds the optimum for MS. As a result, significant amounts of ion–molecule reaction products emerge in addition to monomolecular transformations common in EI-MS. Therefore, the recorded spectra may deviate greatly from library spectra obtained in "conventional" mass spectrometers.

—The mass spectrum depends on the pressure in the ion source, and this pressure varies during the elution of a chromatographic peak. Therefore, the look of a mass spectrum may depend on at what point of the peak the spectrum is recorded: on the upgrade, atop, or on the down grade.

The limited reproducibility of IE-MS influences the identification of stereoisomers (diastereomers). Many of them show but subtle differences in mass spectra, and the reproducibility is insufficient for their reliable discrimination. Therefore, gas chromatographic data (retention indices) are crucial in such cases.

Two-Dimensional and Hyphenated Methods

Typically, a plant extract contains a complex set of metabolites, which cannot be resolved in a single experiment. Even when the sample contains only volatiles, chromatograms can have dense clusters of peaks of individual components with multiple overlaps.

Mixtures containing natural sesquiterpenoids are hard to analyze. Plants usually contain less sesquiterpenoids than mono- or diterpenoids, but the first are of special importance in odor formation [89], in the formation of secondary atmospheric aerosol [90], and as biologically active components of plant extracts [91–100]. Many pairs of sesquiterpenoids have very close or even matching retention indices, and many have practically indistinguishable EI mass spectra [10, 78, 101]. These facts make sesquiterpenoids a very difficult object for analysis [102].

Two-dimensional and hyphenated methods are helpful in the analysis of very complex mixtures of plant substances [103]. Many of them have become important tools for rapid identification of known components in the search for new biologically active compounds [104].

Currently, two-dimensional gas chromatography is one of the most widespread 2D methods. It implies sequential separation in chromatographic columns with different phases [105]. The hybrid method combining 2D GC and MS detection (GC × GC–MS) [5] is of special interest. In this method, the consecutive use of different columns is accompanied by a record of mass spectra of components to facilitate component identification in complex mixtures and increase its reliability. Its potential can be illustrated by analysis of volatiles in tobacco leaf extract [106]. Two-dimensional GC allows enantioselective analysis of components in complex mixtures by using a column with a chiral selector in the second dimension [107, 108]. Collections of experimental data on the elution order of enantiomers under various conditions [109] make the analysis notably easier.

Recent advances in the application of 2D-GC to the analysis of essential oils and aroma substances are reviewed in [7].

Two-dimensional and hyphenated analytical methods often generate bodies of data difficult to process. The application of factor analysis to chromatography by modern hybrid methods is reviewed in [110].

NMR Spectroscopy

Nuclear magnetic resonance spectroscopy is a routine method in the study of organic molecules, including plant substances. Owing to the nondestructive moderate temperature of the analysis, NMR spectroscopy is an indispensable tool in the detection and identification of unstable and thermolabile compounds, such as germacranes [64] and corresponding furanodienes [111], ascaridole [112], acids of the eudesmane family [113], triterpenes [114], and sesquiterpene lactones [115]. In addition, computer-assisted methods based on ^{13}C NMR and data from the literature allow efficient identification of compounds bearing a certain group in plant extracts, as shown by the example of taxanes [116].

Also, NMR spectroscopy is mandatory in the identification of components in very complex mixtures and some particular classes of compounds.

Plant extract profiling. In addition to the common application to the determination of the structure of organic molecules, NMR spectroscopy is used in studies of the chemical compositions of plant tissues and living plants [117, 118] and in studies of metabolite sets regardless of their complexity [119]. NMR allows visualizing profiles of a wide range of metabolites from plant tissues and cells [120]. It makes NMR a promising tool for profiling plant metabolites. NMR provides information inaccessible by other methods [121]. The advance in structural analysis and study of plant metabolomes by high-resolution NMR is summarized

in [122]. Various NMR techniques are greatly helpful in the spectral profiling of medicinal plants and herbal products [3].

Chirospecific analysis. NMR spectroscopy can often efficiently replace enantioselective chromatography. Sometimes, it is the only means to measure enantiomeric purity when enantiomers cannot be separated by chromatography.

The enantiomeric purity of monoterpene derivatives can be analyzed by ^{13}C NMR with the chiral shift reagent Yb(hfc)$_3$ added directly to the sample, as was done with camphor and fenchone [123] and with bornyl acetate [124]. Also, ^{1}H NMR can be applied to isolated compounds, as in a study of linalool [125].

NMR spectroscopy is convenient for analyzing the enantiomeric purity of configurationally unstable compounds. An example is hyoscyamine. It readily racemizes to yield atropine during extraction, and chromatographic methods provide distorted results. The task is resolved by ^{13}C NMR with Yb(hfc)$_3$ [126]. Chiral solvating agents can be used instead of chiral shift reagents, e.g., (*R*)-(–)-2,2,2-trifluoro-1-(9-anthryl)ethanol [127].

Chiral reagents for determining absolute configurations of organic molecules by NRM are overviewed in [128]. The methodology of the determination of absolute configurations is discussed in [129], and recent advances in the NMR-assisted recognition of enantiomers are described in [130].

QUANTITATIVE ANALYSIS

Gas chromatography is one of the few quantitative methods of analysis of complex mixtures. The quantitation of plant extract components demands equipment calibration, measurement error estimation, and the validation of methods for quantitation of analytes.

General issues in quantitation by GC are discussed in the guidebook [131]. Calibration is a procedure aimed at the establishment of the metrological state of a measurement system. Analytical calibration provides an empirical correlation termed metrological function. In subsequent measurements, it allows the amount of an analyte to be deduced from the recorded value of the analytical signal. Analytical calibration is discussed in [132], and quantitative chromatographic analysis by N-dimensional calibration strategies, in [133]. As in any analytical method, quantitative chromatographic analysis encounters the problem of determining the error of measured variables. Sources of systematic errors and methods for their correction are discussed in [134]. The task of error estimation with regard to the methodology of routine analyses is posed in [135–137], where various sides of the search for error sources and appropriate corrections in forming the ultimate measurement result are considered. Any analytical technique requires validation. In [138, 139], issues of analytical method validation are discussed, and a detailed step-by-step guide for this procedure is provided with a description of the most relevant procedures and estimation of measurement errors and accuracy.

Calibration, error estimation, and extraction in chromatographic studies are considered in [140]. A critical review of current methods for quantitation of essential oil components is provided in [141]. Recommendations on the quantitative GC of essential oils and aroma substances are given in [142].

Points of Concern in Quantitation by GLC

Issues of quantitative analysis by gas chromatography have long been known. A number of approaches to quantitative analysis have been developed, and they are considered in detail in special papers. Several guidebooks are available in Russian [143–145]. The analyses often encounter instrumental problems related to signal integration: errors in determining areas of low peaks against noise, irregularly shaped peaks, peak overlay, digitization errors, etc. We will not dwell on these technical difficulties, because their solutions are presented in manuals for gas chromatographic equipment. Instead, we confine ourselves to briefly discussing key questions in quantitative analysis.

Chromatographic quantitative analysis is based on two assumptions: (1) the composition of a sample injected into the chromatograph is the same as in the mixture to be analyzed and (2) the amount of a substance is directly related to the magnitude (area) of the corresponding chromatographic peak. Three methods for quantitation by GC are known.

The absolute calibration method finds an empirical correlation between the area of a chromatographic peak and the amount of the substance injected to the chromatographic column. The method demands a reference, that is, an analyte sample of known purity. In this method, conditions of analysis (chromatography and detection) in calibration and study of an unknown sample should match exactly.

In the internal normalization method, the total area of all chromatographic peaks is taken to be 100%, and the content of an individual component is calculated as the relative area of the corresponding peak. This method is applicable in cases where (1) the chromatogram contains peaks of all components present in the mixture, (2) the detector sensitivity is constant for all components analyzed, and (3) the detector signal linearly depends on the substance amount. In practice, these conditions all together are never met, because almost always some components are invisible, and any detector is selectively sensitive. Therefore, the internal normalization method as it is described provides only a semiquantitative assessment of the contents of components[4]. The simplest way to ensure a reasonable approximation of the result to actual component contents is to apply correcting factors (calibra-

tion factors or detector response ratios), which make allowance for different sensitivities of the detector to sample components. The difficulty is that correcting factors are required for *all components* of the mixture to be analyzed. This requirement can hardly ever be met in practice; therefore, various assumptions and approximations are resorted to.

The internal standard method compares the area of the chromatographic peak of an analyte vs. the area of the peak of an internal standard, which is a known substance whose known quantity is added to the mixture. The analyte concentration is calculated by using correcting factors to take into account different responses of the detector to the internal standard and the analyte. The advantage of this method is that the contents of analytes can be measured without knowing the nature of the rest of the mixture. The disadvantage is the demand for an internal standard meeting a set of requirements, and it is often difficult to choose such a standard for quantitative analysis of complex mixtures.

The standard addition method is employed when no substance can be chosen as an internal standard (see above). A certain amount of a standard analyte sample is added to the mixture to be analyzed, and the content of the analyte is determined by comparison of the original chromatogram and the chromatogram with the additional analyte amount. The method suffers some disadvantages: (1) a standard sample of the analyte or a set of standard samples for quantitative analysis of components should be available; (2) multiple experiments with standard addition of different compounds should be performed to quantitate several components.

Detectors for GLC

Reference data for quantitative analysis of GC may have been obtained with different detectors, and one should always bear in mind features and limitations of this detection type. Correcting factors, which are functions of the response of a detector to a particular substance, are invoked to obtain quantitative data [146]. Response factors obtained for one detector type in different devices vary broadly. Response factors depend not only on the nature of the analyte itself but also on some properties of the analyzed sample and analysis settings. For instance, response factors of various monoterpenes vary with substance concentration, and the concentration functions are different for different monoterpenes, counterintuitively varying from one to another [147]. For the electron capture detector, response factors depend on carrier gas flow in a queer manner [148]. Response factors are unique for this detection method, and they cannot be carried over from one detector type to another. In some cases, response factors are different in different equipment assemblies with identical detector types [149].

Thermal conductivity detector. The thermal conductivity detector (TCD, or katharometer) [150] is inferior to most other detector types in sensitivity, but it is still in common use owing to its universality. The response of a TCD depends on carrier gas flow, carrier gas pressure in the measurement cell, bridge current, detector temperature, and sample mass. With all these parameters precisely controlled, the analysis can be highly accurate and reproducible [151]. It should be remembered that the relative response of this detector depends on the device design; therefore, the calibration of a TCD with reference to reported response factors may cause significant errors in concentration measurement [149].

The calculation of relative molar coefficients for TCD response in gas chromatography has a long history [152]. Analyses of mixtures of fatty acid methyl esters by GLC show that the correcting factors for quantitative analysis in the isothermal and temperature-programmed modes differ [153]. The model of quantitative structure–property relationships (QSPR) can be employed for predicting response factors for TCDs. It has been shown that the application of multiple linear regressions to a certain set of structure descriptors (molecular weight, bond length sum, polarizability, electronegativity, etc.) provides a high correlation between response factors and the set of descriptors. This model allows quantitation of substances for which no experimental response factors are available [154].

Flame ionization detector. The flame ionization detector (FID) is among the commonest detectors in GC [150]. Although it responds to nearly all organic substances, its sensitivity to different classes may vary three to fourfold [155]. It depends on molecular structural features of the compounds [156]. Studies of response factors for 130 organic acid esters (ethyl, isopropyl, *n*-propyl, isobutyl, and *n*-butyl esters of fatty acids C_1–C_{20}, aliphatic dicarboxylic acids C_2–C_{12}, benzoic acid, and *o*-phthalic acid) showed that they varied over a broad range: from 1.10 in ethyl esters of fatty acids to 3.67 in *n*-butyl phthalate [157]. Certain difficulties arise in the quantitative analysis of lower fatty acids as methyl esters; therefore, it is sometimes more convenient to analyze them as heavier esters, e.g., butyl [158]. Response factors were measured for ten monoterpenes with *p*-cymene as an internal standard and proved to be within 0.844–1.055. Exceptions were limonene (0.938–1.266) and myrcene (0.681–0.813). The underestimation of myrcene concentrations may be related to its tendency for dimerization to dimyrcene (camphorene) and polymerization at temperatures above 200°C [147]. In analyses of complex mixtures of terpenoids diverse in structure and properties and having different response factors, all compo-

[4] For this reason, special journals with an increased focus on the analytical side, such as *The Flavour and Fragrance Journal,* refuse to accept papers in which compositions of volatiles and essential oils are obtained by simple internal normalization.

nents are divided into groups of similar substances and calibration is done for one component from each group; e.g., 2-carene for monoterpenes and carvone for simple oxidized derivatives [159]. This procedure lowers the detection limits and provides a satisfactory accuracy of quantitation.

Several approaches to predicting FID responses are known. One of the first, which is still in progress, is based on the concept of effective carbon number [157, 160–164].

Another approach rests on the fact that analytes burn during FID operation; hence, response factors may depend on their combustion heats. Indeed, there is a good correlation between response factors and combustion heats [165]. As the combustion heats of organic substances can be predicted by quantum chemistry methods within a reasonable accuracy, response ratios of FID can be efficiently predicted, as shown by examples of volatile components of various fragrances [166]. Analysis of silyl derivatives of biomolecules showed that the prediction of response factors on the basis of predicted combustion heat values was among the few methods allowing quantitation of volatiles without device calibration with pure reference compounds [167].

Still another approach utilizes molecular descriptors such as the inner molecular polarizability index ($IMPI_m$) [168]. It was attempted to model FID response factors by using an artificial neural network, and the designed network demonstrated a satisfactory predictive force [169]. Multiple linear regression with an artificial neural network involving several molecular descriptors (molecular weight, number of vibrational modes, surface area, and the Balaban index [170]) provided a good prediction of response factors for various classes of compounds [171].

It has been shown that the helium pulsed-discharge photo ionization detector (PDPID) is more accurate than FID in determining the percentages of hydrocarbons in a mixture. In addition, the former is more universal [172].

Mass-selective detector. The most numerous difficulties arise in using the mass spectrometer as a detector for quantitative analysis [68, 88, 173]:

—The sensitivity of the mass-spectrometrical detector in the record of a full mass spectrum is generally notably lower than that of FID, and it greatly depends of the injection method [174].

—As the dynamic range of the detector is finite (about 10^6), peaks of major components in complex mixtures are distorted and low-intense components are masked with noise. Both these facts generate significant integration errors.

—The speed of the operation of the mass-analyzing magnet is finite (Modern devices with the quadrupole mass spectrometer analyze two to ten spectra per second.) As a result, the digitization of a chromatographic peak is inaccurate, and the integral intensity is determined with a significant error.

—All other conditions being equal, the ion flow is determined by ionization cross-section, which depends on the broadly variable molecular structure (shape). It means in practice that two compounds having equal ionization cross-sections and entering the ionization chamber in equal quantities produce different amounts of ions and, consequently, different ion flows and different areas of their chromatographic peaks.

—A mass analyzer of any type transmits ions differently with different m/z values. Therefore, when equal numbers of ions with two different masses emerge in the ionization chamber, it does not necessarily mean that ion flows corresponding to these masses in the mass-selective detector are equal. The difference in ion flows is the greater the greater is the difference in m/z values. Different mass analyzer types have specific features in this regard. Owing to the specific location of the secondary electron multiplier in the quadrupole analyzer, the multiplication factor for heavy ions is less than for light ones.

—Generally, for practical purposes, not the whole mass spectrum is recorded, and ions with m/z less than 30 Da are cut off. Thus, two substances with equal ionization cross-sections entering the ionization chamber in equal quantities produce different integral ion flows in detection, and the difference in intensity is the greater the greater are differences in the proportions of the lightest and heaviest ions for these substances.

As in the use of other detector types (see above), the application of the mass-selective detector to volatile quantitation requires knowledge of the response factors of particular components. Studies of the dependence of the mass-spectrometrical detector response on molecule structure show that this detector is generally less sensitive to *n*-alkanes than to corresponding halogenated derivative, and polyhalogenated alkanes demonstrate a significant departure from the additivity rule [175].

Standards and Standard Mixtures

Quantitation by gas chromatography requires standards, which are substances of known purity or mixtures of known compositions suitable for calibration. Any substance meeting the following requirements can be used as a standard: (1) chemical similarity between the standard and compounds analyzed for the standard being completely miscible with the sample and for the chromatographic behaviors of the standard and compounds analyzed being comparable; (2) storability and stability under analysis conditions; (3) inertness to all components of the mixture to be analyzed; (4) purity determined by an independent method and freedom from impurities whose peaks can

overlap those of the mixture to be analyzed [142, 144]. For some mixtures, it is not so easy to choose a standard that would meet all these requirements [176].

The analysis and purity check of such standards and mixtures for calibration are a special task, sometimes difficult. Various approaches to such analyses are described in [177, 178]. There are methods saving time for the preparation and analysis of standard mixtures [179]. A protocol for preparation and use of standards with concentrations within 1–10 ng/μL for GLC quantitation of mono- and sesquiterpenes in biologic fluids is proposed in [180].

For a variety of causes, many compounds are unavailable as standards for quantitative analysis, and in such cases, ambiguity is unavoidable. However, the group-correlation method allows mitigating this uncertainty and accurately quantitating mixtures containing one or more unavailable components [181].

Quantitative NMR Spectroscopy

In contrast to chromatographic methods, quantitative nuclear magnetic resonance (qNMR) spectroscopy is a universal method for detection and quantitation of analytes in mixtures as complex as they can be [182]. In the analysis of plant extracts, qNMR is apparently advantageous over common methods based on chromatographic resolution [183]. The decisive advantage is that qNMR measurements do not require pure authentic standard samples of analytes. The analysis can be done with any available pure sample of an organic compound as an internal standard [184]. Another feature of NMR is that quantitative measurements of several components can be done in parallel [185] with a single standard [186]. It is possible to conduct qNMR measurements even without any standard when concentrations are calculated with reference to solvent signals [186].

ANALYSIS OF SPECIFIC TYPES OF SUBSTANCES

Each type of volatile plant metabolites demands special choice of the method and conditions for analysis with regard to physical, physicochemical, and chemical properties of the compounds. Specific features in analysis of various compound types by GC–MS are described in [10, pp. 172–180]. To avoid duplicating this guidebook, we confine ourselves to data omitted from it or obtained after publication.

Normal Hydrocarbons

Normal hydrocarbons are a widespread group of compounds obtained from living plants, plant material, and fossil sources. Mixtures of normal alkanes are ubiquitous components of plant epicuticular wax [187]. Normal hydrocarbons are used as universal standards in the calculation of retention factors in GC and in the calibration of columns for gel permeation chromatography to determine important parameters of oil fractions [188]. Normal alkanes are present in all oil derivatives, fuels, and lubricants. Therefore, they often pollute plant extracts.

The analysis of mineral oils and their fractions is of special importance in chromatographic studies because many key items of qualitative and quantitative chromatographic analysis have been investigated by the example of oil fractions. Studies of oil fractions generated interesting approaches associated with the simulation of distillation results in quantitative analysis and use of correction factors [189].

Fatty Acids and Their Esters

Fatty acids are among the main intermediates in primary metabolism. As a rule, they are not accumulated in plants in a free form, rather, they are deposited as triglycerides (fats) and involved in the synthesis of various lipids [190]. Free fatty acids serve as signaling molecules and, as such, participate in the regulation of some enzymatic pathways [191]. Small amounts of free fatty acids are present in plant epicuticular wax [187, 192].

Instead of direct analysis of fatty acids, experimenters usually convert them to methyl esters. Methods for the analysis of the latter have been well developed, and the compositions of fatty acid fractions can be determined in products from various sources [193]. Nowadays, the chromatographic study of fatty acid methyl esters attracts great attention in connection with the analysis of biodiesel [194–196], an increasingly popular fuel.

Essential Oils

The application of chromatographic methods to the study of the compositions of essential oils has been greatly developed. The current state of the methodology of essential oil extraction and analysis is reviewed in [70]. The quantitative analysis of essential oils is still difficult because most components are complex in structure and unavailable as calibration standards. It was found that quantitative data on essential oil composition obtained by GC–MS without correction for response factors of particular components were of very poor accuracy [197]. Recent advances in the instrumentation for the chromatographic analysis of essential oils are discussed in [4–7].

Owing to plant polymorphism and various ambient factors, essential oil yields and compositions may be widely variable within a single plant species [198–200]. The composition of essential oil from a plant species obtained in an experiment as compared to reported data may astonish the scientist. The mismatch is associated with at least two factors: the already mentioned natural variability of plants and differences in protocols of processing and analysis, gen-

erating different sets of artifacts. In such cases, comparison with standard chromatographic profiles for species at issue (chemotypes) can be helpful. Unfortunately, standard chromatographic profiles are available only for commercial essential oils. As for wild plants, the only summary on odoriferous plants of southern Siberia was published in [14].

Terpene Hydroperoxides

We have already mentioned that products of terpene oxidation during storage of essential oil are potent allergens. In this regard, their contents in terpene-containing products attract special attention. The quantitation of terpene hydroperoxides is a difficult task, because conventional chromatographic procedures require standard samples of limited accessibility. Moreover, they should be stored under drastic conditions (–78°C) because of their chemical instability. To overcome this difficulty, an approach based on GC–FID analysis of silylated hydroperoxide derivatives with response factor prediction has been proposed [201]. This procedure is a satisfactory alternative to full calibration at concentrations below 500 ppm, although large deviations are observed in analyses of essential oils and artificial perfumeries. These deviations are related to the instability of hydroperoxides, easily reacting with other components, rather than to procedural errors. Thermolability is another source of errors in the GC analysis of hydroperoxides because heating is indispensable in GC studies. To eliminate this difficulty, a simple and sensitive method for quantitation of hydroperoxides in linalool, linylal acetate, and limonene was developed on the base of tandem liquid chromatography–mass spectrometry (LC/ESI-MS/MS) [202].

Flavoring Substances

As essential oils and extracts are important ingredients of flavorings, the requirements on the trade of natural materials used for this purpose tend to toughen [203]. In this regard, new approaches to proximate analysis of volatiles in various aromatizing agents without authentic standards are being developed to avoid labor-consuming calibration [204].

In spite of the ubiquitous use of natural fragrances and flavorings, one often loses sight of the fact that not all natural components are harmless. Many natural mixtures used to flavor various products are fraught with substances whose contents should not exceed certain permissible limits because of their toxicity. Although this was known for a long time, the first comprehensive analytical study on this topic was published no sooner than 2015, when the workgroup on analysis methods, the International Organization of the Flavor Industry (IOFI), elaborated a procedure for rapid routine GS–MS assay of substances of this sort: β-asarone, coumarin, menthofuran, methyl chavicol, methyl eugenol, pulegone, safrole, and α- and β-thujones. The method was tested in several laboratories and proved to be efficient [205].

Sesquiterpenoids. As noted above (see section Two-dimensional and hyphenated methods), sesquiterpenoids are difficult to analyze. Although the identification of some widespread compounds (caryophyllene\isocaryophyllene, humulene, longifolene, germacrene, caryophyllene oxide, spathulenol, bisabolol, etc.) by GC–MS is simple, the overwhelming majority of sesquiterpenoids present problems because of the similarity of their retention indices and mass spectra. Just for this reason, in order to avoid misidentification, the authors' guide of the specialized *Flavour and Fragrance Journal* notes that the match of retention indices and electron impact ionization mass spectra is insufficient for the identification of unusual sesquiterpenoids; thus, the identification should be proven by additional spectroscopic methods, GLC–IR and/or NMR.

The quantitation of sesquiterpenoids is also a complex task, because most compounds of this group are unavailable as analytical standards.

Sousa et al. reported an example of the development and validation of analytical protocols for quantitative assay of key sesquiterpenes—β-caryophyllene, α-copaene, and α-humulene—present in extracts from plants of the genus *Copaifera* (family Leguminosae = Fabaceae) with 1,2,4,5-tetramethylbenzene as an internal standard [206]. Many practically important sesquiterpenoids are multiple-function compounds, unstable under GC conditions. Liquid chromatography with a single standard, curdione, allows quantitation of key sesquiterpene derivatives present is preparations obtained from curcuma (*Curcuma* spp.): zedoarondiol, isozedoarondiol, aerugidiol, (4*S*,5*S*)-(+)-germacrone-4,5-epoxide, curcumenone, neocurdione, germacrone, and furanodiene [207].

Analysis of Biogenic Volatiles in the Air

Volatile organic compounds are important for atmospheric chemistry and biogeochemistry. Biogenic emissions constitute the greatest portion of volatile atmospheric organic substances other than methane. In their analysis in the atmosphere, GC–MS has a series of advantages over other analytical methods [208]. However, precise quantitative calibration requires standards, which are not always available. A special feature of the analysis of monoterpenes in the air is the demand for standard mixtures of very low concentrations. Protocols for preparation of such mixtures with concentrations about 2 nM are described in [209].

A seven-parameter QSPR model was developed in [210] to predict response factors of the mass-selective detector for various chemical structures. The mean error in the prediction of response factors was calculated by the cross-check procedure to be below 20%,

which is satisfactory for an air volatile assay. Also, a method for the quantitation of biogenic volatiles in the air with FID employing the notion of effective carbon number has been proposed [211].

In some cases, constant monitoring of biogenic organic substances in the air demands a better time resolution than in routine analyses. For this reason, a completely automated method of fast gas chromatography was developed to ensure closer control of the time resolution of monoterpenes and some other terpenes C_9–C_{15} [71]. Reasonable accuracy with errors not exceeding 12% for monoterpenoids and 25% for oxidized sesquiterpenoids was achieved within 10–20 min.

CONCLUSIONS

As a result of the widespread occurrence of modern GC instrumentation equipped with up-to-date computerized tools for experimental data collection, preprocessing, and analysis, a great portion of experimental work on component identification and quantitation, starting from sample injection to the reading of data, is automated. Thus, the analysis of volatile and semivolatile components of plant extracts becomes a pleasure cruise. It should be kept in mind, though, that the apparent simplicity of routine analyses characterizes only technicality. Indeed, the modern hard- and software considerably facilitate measurements and minimize manual operations. Nevertheless, the improvement of experimental techniques does not change the basics of a method, and its intrinsic limitations persist. Therefore, chromatographic or GC–MS data obtained in the automated mode should undergo unbiased revision by the experimenter to avoid misinterpretation in publications. Proper interpretation of data demands that the entire history of the sample be known and all manipulations with plant material from collection to final sample preparation be most carefully documented. Otherwise, we cannot expect meaningful results.

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